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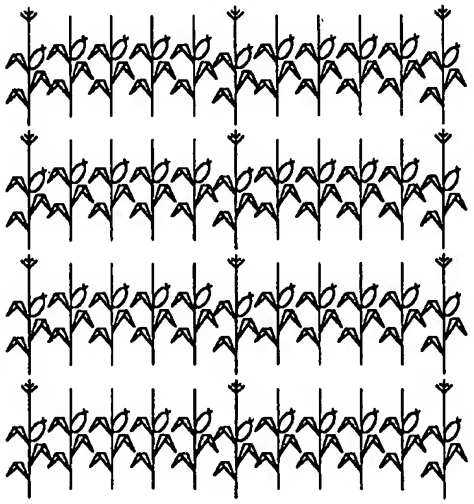
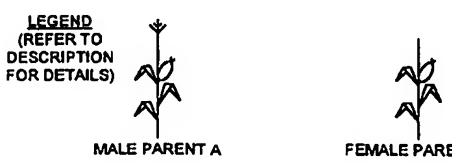
(54) Title: PROTEIN COMPLEMENTATION IN TRANSGENIC PLANTS

(57) Abstract

The invention relates to pairs of parent plants for producing hybrid seeds and to methods for producing plants with a desired phenotype. The desired phenotype is an active enzyme, a regulatory protein or a protein which affects the functionality and/or viability and/or structural integrity of a cell. Preferably, the desiredphenotype is substantially absent from the parent plants/lines. In particular, the invention relates to parent plants and methods involving plant lines for producing male–sterile plants and seeds.

FIGURE SHOWING A PRODUCTION SCHEME OF EMBRYO LESS MAIZE GRAINS: LINES A AND B ARE SOWN IN ALTERNATIVE ROWS (FOR EXAMPLE ONE MALE AND FOUR FEMALES)





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# PROTEIN COMPLEMENTATION IN TRANSGENIC PLANTS

This invention relates to pairs of parent plants for producing hybrid seeds and to methods for producing plants with a desired phenotype. The desired phenotype is an active enzyme, a regulatory protein or a protein which affects the functionality and/or viability and/or structural integrity of a cell. Preferably, the desired phenotype is substantially absent from the parent plants/lines. In particular, the invention relates to parent plants and methods involving plant lines for producing male-sterile plants and seeds.

The present invention describes a protein complementation system, with a variety of different applications. The system can be explained and exemplified with reference to obtaining male-sterile plants and embryoless seeds although it is not limited to these applications.

The use of dominant Artificial Male Sterility (AMS) in plants is described in WO95/20668. This document describes a binary system using two genes which together (but not in isolation) cause male sterility. The genes are brought together by crossing plants, each parent being homozygous for the gene, which generates a homogenous population of male sterile plants. WO95/20668 describes several ways to implement the gene binary system, including the following:

i. a system based on activation of transcription: a transcriptionally inactive AMS gene is activated upon crossing by provision of the relevant transcription factor;

ii. a system based on activation of splicing: an AMS gene inactivated by the presence of an intron is activated upon crossing by provision of the relevant maturase;

iii. a system based on the suppression of a stop codon during translation: an AMS gene inactivated by introducing an artificial stop codon in the ORF, is activated upon crossing by provision of an artificial stop suppressor tRNA for the introduced stop codon.

iv. a system based on sequence-specific gene inactivation: One parent contains a modified male fertility gene and a transgene which inactivates only the unmodified male fertility gene. The other parent contains a transgene which inactivates only the modified male fertility gene. In the hybrid both the modified and unmodified male fertility genes are inactivated causing male sterility.

v. a system based on preventing restoration of male fertility by a restorer gene: the first parent contains the AMS gene and the restorer gene, and the second parent contains a gene inhibiting the action of the restorer gene.

However, the binary systems described above have so far proved complex to implement and have encountered a variety of difficulties.

For example, it has been found that the use of a suppressor tRNA (described in Betzner *et al.* 1996,

Abstract of the 14th International Congress of Plant Reproduction, Lorne, Australia) can have deleterious consequences for some plant species. While this does not preclude its use, it does make the screening of suitable transgenic plants more labour intensive than desirable. Another example is the leakiness of the T7 promoter (described in EP-A-0589841). Some plants transformed with a T7 promoter driving Barnase were sterile in the absence of the T7 RNA polymerase. Again, this does not preclude use of the system but it does make it difficult to identify suitable transgenic plants. Furthermore, in certain plants the gene binary system is sub-optimal since not all of the required genetic elements are fully characterised.

Two areas of prior art have been explored which have resulted in a phenotype conferred to a plant by the combination of two proteins.

In 1989, Hiatt and coworkers (*Nature*, vol. 342, p. 76-78) described the production of a functional antibody in tobacco by crossing tobacco plants expressing a gamma immunoglobulin gene and a kappa immunoglobulin gene.

Problems were, however, encountered with this system. Since the light and heavy chains of an antibody interact through disulfide bridges, the bridges were unable to form in the reducing environment present in the cytoplasm. Assembly of a functional antibody in plants thus requires that both chains are targeted to the endoplasmic reticulum then secreted to the apoplast (the space between cells). The production of antibodies in plants has thus been limited to the production of secreted antibodies or the production of single chain

antibodies.

In 1992 Lloyd *et al.* (*Science*, vol. 258, p. 1773-1775) described the transfer in Arabidopsis and tobacco of two maize genes coding for the transcription factors R and C1. Ectopic expression of these genes separately in heterologous plants has some effect on the transcription of endogenous genes. In particular the genes have some effect in isolation, and this may preclude their use for applied purposes. Co-expression of the two genes had more dramatic qualitative and quantitative effects, than expression of either gene alone. However, these genes have properties severely limiting their usefulness and their general inapplication is described in the paper.

It has been shown that the Arabidopsis transcription factors Apetala3 and Pistillata can be ectopically co-expressed, and jointly in concert cause a new phenotype in the Arabidopsis flower (Krizek and Meyerowitz, 1996, *Development*, vol. 122, p. 11-22). The limitations described above for the R/C1 proteins also apply in this case.

The present invention describes a protein complementation system which overcomes many of the problems and difficulties associated with known gene binary systems. The protein complementation system according to this invention is based on the expression of two or more gene sequences in a single plant, which polypeptides/proteins, associate, interact or come together to form an active enzyme, a regulatory protein or a protein which affects the functionality and/or viability and/or the structural integrity of a cell. Hereinafter, in this text all references to a protein

which affects the structural integrity of a cell also describes a protein which may, in addition, or alternatively, affect the functionality and/or viability of a cell. Some polypeptides/proteins may fall in more than one of these categories None of the individual gene sequences present in a given plant lead to a significant phenotypic effect in these plants.

The present invention describes the creation of a plant which has a desired phenotype through expression of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell (eg. a membrane destabilizing protein). The plant may be obtained by crossing a pair of parent plants a and b. Plant a contains one or more gene sequences which encode a polypeptide(s) or protein(s) (A) with little or no activity so that the desired phenotype is not significantly (or substantially) caused by expression of the one or more genes in plant a alone. Plant b also contains one or more gene sequences which encode a polypeptide(s) or protein(s) (B) also, with little or no activity so that the desired phenotype is not significantly (or substantially) caused by expression of the one or more genes in plant b alone. When plants a and b are crossed, the resulting hybrid expresses both polypeptides and/or proteins A and B. These two polypeptides/proteins associate, interact or come together to form an active enzyme, regulatory protein or protein which affects the structural integrity of the cell, with the result that the daughter plant displays the desired phenotype. NB: From hereon, when discussing the polypeptides/proteins A or B they will be referred to only as 'polypeptides' for the sake of convenience.

This protein complementation binary system is simpler than the previously described binary systems since there is no need for interaction between genes, no required modification of the expression of genes and no modification of the level of expressed polypeptides in the daughter plant compared to the parent plants.

The present invention is described with reference to the Figures which are:

FIGURE 1A; Barnase coding sequence;
FIGURE 1B; Intergenic sequence;
FIGURE 1C; Barstar coding sequence;
FIGURE 1D; Translational fusion of ORF Peptide A**/(Gly4 ser)3 Linker peptide / GUS;
FIGURE 1E; Nucleotide sequence of Translational fusion of Ubiquitin genomic sequence and ORF Peptide A***;
FIGURE 1F; Nucleotide sequence of Translational fusion of Ubiquitin genomic sequence and ORF peptide B***
FIGURE 1G; DNA sequence of IPCR (inverse polymerase chain reaction) primers (example 1)
FIGURE 2; Schematic illustration of pepA* and pepB* construction by Inverse PCR (IPCR)
FIGURE 3A; *In vitro* construction from synthetic oligonucleotides of S-peptide, S(+5)-protein and S-protein;
FIGURE 3B; *In vitro* construction from synthetic oligonucleotides of the sequence encoding the S-peptide and the (Gly4-Ser)3 linker;
FIGURE 4A; protein and DNA sequences of S-peptide and S-peptide with (Gly4-Ser)3 linker;
FIGURE 4B; protein and DNA sequences of S(+5)-protein and S-protein.
FIGURE 4C(i); PCR amplification product encoding partial

AOX3 targeting signal;

(ii); ORF encoding AOX3 targeting sequence (underlined) and S-peptide

(iii); ORF encoding AOX3 targeting sequence (underlined) and S-peptide/(Gly4 Ser)3/GUS

(iv); ORF encoding AOX3 targeting sequence (underlined) and S-protein

(v); translational fusion of Ubiquitin genomic sequence and ORF of S-protein;

FIGURE 4D; nucleotide sequence of IPCR primers (example 3)

FIGURE 5; production scheme for embryoless maize grains.

Embroyoless seeds harvested from female rows only = 100% of embryoless maize seeds

or

Seeds harvested from all the field plants = approximately 80% of embryoless maize seeds:

note that if this sort of seeds harvesting is suited a random sowing with 10% of male plants and 90% of female plants is desirable and possible.

Legend

male parent A
expressing pepA* in embryos
Genotype: emb-pepA*/emb-pepA*
or
emb-pepA* linked to Herbicide resistance/emb-pepA* linked to herbicide resistance

female parent B
expressing pepB* in embryos only
Genotype: emb-pep*/emb-pepB* in a
male sterile cytoplasmic environment
or
emb-pepB*/emb-pepB*
Artificial Male Sterility linked to
Herbicide Resistance/+

According to a first aspect of the invention there is provided a pair of parent plants for producing seeds comprising:

(i) a first parent plant containing one or more gene sequences encoding a polypeptide A; and
(ii) a second parent plant containing one or more gene sequences encoding a polypeptide B;

wherein the polypeptides A, B, when expressed separately in different plants, do not form an active enzyme a regulatory protein or other protein which affects the structural integrity of the cell but when expressed in the same plant do form an active enzyme, regulatory protein or other protein which affects the structural integrity of the cell. Presence of the active enzyme, regulatory protein or protein which affects the structural integrity of the cell in a single plant, is the desired phenotype.

The present invention includes the scenario of inter-

extra-genic repression/complementation/suppression; that is, where a mutation in one subunit of a multi-subunit complex can complement a mutation in another sub-unit in order to restore the active enzyme, regulatory protein or protein affecting the structural integrity of the cell. In such a scenario, the polypeptide(s)/protein(s) A and B may be the same in the two parent plants, with the exception of the different mutations. Examples include the *E.coli* regulatory proteins as described by Tokishita S.I., and Mizuno T., 1994, *Mol. Microbiol. (UK)*, 13/3, 435-444 and the GroES and GroEL proteins of *E.coli* as described by Zeilstra-Ryalls J., *et al.*, 1994, *J. Bacteriol. (US)*, 176, (21), 6558-65.

In the present invention, the pair of parent plants can be described as a pair of complementary plants for producing hybrid seeds or even a pair of complementary transgenic plants for producing transgenic hybrid seeds.

It is most likely that at least one of the pair of parent plants is transgenic. When used herein the term 'transgenic' refers not only to genetic material from another species but to genetically manipulated DNA from the same plant or species. The genetic manipulation of the plant may be by a microbiological process such as *Agrobacterium tumefaciens* (Horsch R.B., Fry J.E., Hoffman N.L., Eichholtz D., Rogers S.G., Fraley R.T., (1985), *Science*, 227 : 1229-1231)). Alternative manipulations include biolistic transformation, a technique also well known in the art, the use of *Agrobacterium rhizogenes*, particle gun, electroporation polyethylene glycol or silica fibers.

The present invention may be applied to any plant, in

particular, maize, wheat, tomato, oilseed rape, barley, sunflower, linseed, peas, beans, melon, pepper, squash, cucumber and egg plant (aubergine) and other broad acre plants.

Use of the term "one or more gene sequences encoding a polypeptide...." refers to any number of stretches of genetic material (preferably DNA) which can encode one or more peptides/polypeptides/proteins. Thus "polypeptides" A or B can actually comprise more than one amino acid sequence which may or may not be linked or associated. There is no restriction on the location in the parent plant genome of the one or more gene sequences. Where more than one gene sequence is present, encoding for more than one peptide/polypeptide/protein, the relationship between the encoded sequences in each parent plant is only relevant to the extent that the parent plant does not display the desired phenotype (to any significant level). When the one or more gene sequences encoding a polypeptide A are expressed in the same plant as the one or more gene sequences encoding polypeptide B, then the result, according to the invention is the phenotype of an active enzyme, a regulatory protein or a protein which affects the structural integrity of a cell. Proteins which affect the structural integrity of a cell include proteins that destabilise or create holes or ion channels in cellular membranes.

A particular application of the present invention is the production of male-sterile plants. Accordingly, the polypeptides A, B when expressed in the same plant may cause male-sterility by ablation of the tapetum. An alternative application, also of the first aspect of the

invention is the expression of polypeptides A, B in the same plant which form an active enzyme, a regulatory protein or protein which affects the structural integrity of a cell, which, through cell ablation in a specific tissue results in a different phenotype, as described below.

In addition to causing male-sterility, potent hydrolases like Barnase can be used for other applications where cell ablation is needed, for example to remove an unneeded organ from a hybrid crop. This may contribute to reducing downstream processing costs. One example is the production of embryoless seeds, which is now described as follows: In the production of flour (from wheat) or semolinas (from maize or wheat) or corn flakes (from maize) or for other uses, it would be desirable to have seeds with no embryo. The use of embryo specific promoters in the first aspect of the invention above would enable ablation of embryos in seeds, in a cross dependent manner. That is, in the seeds produced by the plant containing one or more gene sequences encoding polypeptide A, pollinated with pollen from a plant containing one or more gene sequences encoding polypeptide B. Self pollination of plant a has to be prevented, for example by making plant a male-sterile. A possible production scheme for valuable embryoless maize grains would be the following: generate a plant containing one or more gene sequences encoding polypeptide A (plant a) and a plant containing one or more gene sequences encoding polypeptide B (plant b), designed so that combination of polypeptide A and polypeptide B in one seed results in embryo ablation. Figure 5 shows a production scheme for embryoless maize grains according to the invention.

The biochemical composition of plants can also be manipulated according to the first aspect of the invention, for example by fatty acid biosynthetic enzymes. Where the presence of an unusual but valuable fatty acid in the plant has a deleterious effect on the plant, it would be useful to be able to produce seeds with the unusual (fatty acid) oil through a cross between two lines having a normal (or quasi normal) oil composition (to the extent that each parent line is not deleteriously effected). Splitting the enzyme responsible for the valuable fatty acid biosynthesis in two or more inactive parts, provides a practical way of producing the seeds with the valuable oil. Where the enzyme responsible for the desired trait is heteromultimeric, separating the genes from the various monomers in the two parent plants is a simple way to implement the invention. More generally, this invention can be used to obtain hybrid seeds or hybrid plants with a particular phenotype which neither parent has. In particular, this invention can be used to create hybrid plants, resistant to a herbicide, via the crossing of two parent plants. Each of the parent plants expresses one or more non-functional parts of an active enzyme, regulatory protein or protein which effects the structural integrity of a cell, which is directly or indirectly responsible for herbicide resistance. As the one or more genes in each parent plant responsible for the trait will segregate independently, this will result in the gametes of such hybrid plants (especially pollen grains) giving rise to a lower transfer of the herbicide resistance trait to relatives or to weeds (in comparison with a classical single gene). If the hybrid seed is the harvested desirable product, expression of the

desired trait would be restricted to the seed endosperm and embryo since these tissues are genetically hybrids.

The active enzyme, regulatory protein or protein which affects the structural integrity of a cell is preferably localised to a tissue specific (ie. present only in a selected tissue). This requires that one or both of the gene sequences encoding the polypeptides A, B are operatively linked to an appropriately stimulated promoter, eg. a tissue specific promoter so as to produce the desired phenotype. Where only one of the polypeptides is limited to expression in a selected tissue, the other polypeptide requires constitutive expression or at least an expression pattern which overlaps with that of the first polypeptide.

As described above, the expression may be seed or embryo specific and promoters for such tissue specificity are well known in the art. In the case of male-sterility, the promoter is preferably tapetum specific. Such promoters known in the art include the TA29 promoter (EP-A-0344029), the A9 promoter (Paul *et al* 1992, Plant Molecular Biology, vol. 19, p. 611-622) and the promoters described in WO95/29247. In order for heterozygous plants to have the desired phenotype, promoters must be active at the sporophytic level.

The choice of gene sequence for producing an active enzyme, regulatory protein or protein which affects the structural integrity of a cell depends, of course, on the desired phenotype. Any gene sequence encoding an active enzyme, regulatory protein or protein which affects the structural integrity of a cell can be used provided that the protein activity can result from the

association, interaction or combination of two or more polypeptides encoded by two or more gene sequences and that their activity can result in the desired phenotype. Immediately obvious proteins which can be suitable are those which are naturally encoded by two or more polypeptides and which self-assemble to form the final protein structure. The individual polypeptide units (subunits) should have no significant activity *in vivo*.

Suitable proteins for use according to the invention include natural heterodimeric proteins such as the C1-R maize proteins and the Apetala3-Pistillata (Ap3-Pi) *Arabidopsis thaliana* proteins. When present in the tapetum, the dimer protein Ap3-Pi can activate genes responsive to this transcription factor (which would normally be inactive because this transcription factor is normally absent from, or present at a low level in, the tapetum). The activated gene is preferably, but not necessarily, endogenous to the plant of interest. For example, expressing the dimer Ap3-Pi in the tapetum of maize will activate transcription of genes normally involved in flower development in other floral organs, and will prevent normal pollen maturation. The level of sterility of such a system can be improved by also engineering into the daughter plant a gene sequence which is affected by the produced active enzyme or regulatory protein.

One example is the introduction into one of the parent lines of a gene sequence from Barnase or PR-Glucanase under the control of the Apetala3 promoter (pApetala3). The Apetala3 promoter is responsive to the Ap3-Pi dimer and thus expression of the Barnase or PR-Glucanase protein occurs in the daughter plant. Such a system

provides for the enhancement of plant male-sterility with the additional advantage of being under a strict control mechanism (via the pApetala3). Thus, the cause of the desired phenotype may be direct, ie. a direct result of the active enzyme, regulation protein or protein which affects the structural integrity of a cell, or may be indirect, ie. acting via an intermediate factor. Other transcription factors, for use in the invention, exist already as, or can be engineered to, a heterodimeric form, for example using the dimerisation domains described below. These include artificial transcription factors made by the association of a DNA binding domain and an activation domain of different origins.

An alternative use of the Apetala3-Pistillata system, is the complementation of mutations in sub-units of the proteins. For example, one parent plant may express both proteins but with a mutation in one or the other so that the plant does not have the active dimer. The other parent plant may also express both proteins, in this case, a mutation being in the other protein. The second parent plant would not express the active dimer. A cross between the two parent plants would result in expression of genes to produce an active dimer.

Ectopic expression of the subunits for these transcription factors can be used to modulate expression of their target gene and cause male sterility or other traits (including pleiotropic effects) in a cross-dependent manner.

It is also possible to use, according to the first aspect of the invention proteins which have to be

"artificially" split into two or more nucleic acid coding sequences. The resulting polypeptides/proteins must associate, assemble, interact or come together when expressed in the same plant to form an active enzyme, regulatory protein or protein which affects the structural integrity of a cell. Such artificial splitting of enzymes and proteins is today easily achieved by predicting where the protein can be split into two or more domains, for example predicting by structural biochemistry such as X-ray crystallography, functional protein analysis in mutants, structure prediction from sequence analysis or by limited proteolysis, amongst other techniques. In this way, the random coil or other suitable regions are identified as places where the protein may be split.

Examples of artificially split proteins include:

Barnase: This protein has been widely used to cause cell ablation, when expressed in specific tissues. Under the control of a tapetum specific promoter, expression of a Barnase gene causes male-sterility in many plant species (EP-A-0344029). It is known that the Barnase protein can be split into two polypeptides, which *per se* have no catalytic activity [*in vitro*]. When put together the two polypeptides can self-assemble to produce an enzyme whose product has RNase activity. (Sancho and Fersht, 1992, J.Mol.Biol., 224, 741-747).

RNase A can also be used. It was shown, as long ago as 1959 (Richards and Vithayathil, J.Biol.Chem., 234, 1459-1465) that RNase A can similarly be split by mild proteolytic treatment into two polypeptides which can then reassociate and produce an active enzyme.

In order to implement a system, according to the present invention, involving artificially split proteins, it may be necessary to design genetic constructs in order to express the polypeptides therefrom. In order to design the genetic constructs whose products will associate to form the active enzyme some modifications may be required. For example,a methionine codon can be added in front of the ORF encoding the second half of the active enzyme and a stop codon can be added after the ORF encoding the first half of the active enzyme. If the polypeptides are expressed as the C terminal part of a translational fusion to another protein or to a protein targeting sequence, then a start codon may be absent from the ORF of polypeptide A and/or polypeptide B, whereas a stop codon is still needed to terminate the ORF of polypeptide A and polypeptide B, respectively. If polypeptide A or B is expressed as the N-terminal part of a translational fusion to another protein, then the ORF of polypeptide A or B will start with a methionine codon whereas the termination codon is provided by the ORF of the other protein to which it is fused. Such genetic construct design is commonplace and well known to the person skilled in the art.

The invention may also be practised by expressing two portions of two different enzymes that together give a different activity than either of the intact parent proteins.

Preferably, both parent plants are homozygous with respect to the gene sequences encoding polypeptide A or polypeptide B. Such genotypes ensure that all offspring will express the active enzyme, regulatory protein or

protein which affects the structural integrity of the cell.

If one or more of the polypeptides (A or B) is/are small and there are doubts that any of them will be stable in a cell, it is possible to use well-known systems wherein the small polypeptide is fused in frame to a "carrier protein" which protects it from being degraded or increases its proteolytic stability, but retains its freedom to interact with the other polypeptide(s) to form the active enzyme, regulatory protein or protein which affects the structural integrity of a cell.

The carrier protein can be chosen so that the polypeptides A or B are not affected by the fusion. One suitable carrier protein is the β-Glucuronidase (GUS) protein, which tolerates addition to its $NH_2$ end, and is a good reporter gene in plants. In this case, one can use the level of GUS activity to evaluate the expression level of the fused small polypeptide. This can be useful for screening primary transformants. Another suitable carrier protein is ubiquitin (Hondred and Vierstra, 1992, Curr. Opin. Biotechnol. 3, 147-151; Vierstra, 1996, Pant Mol. Biol., 32, 275-302). When fused in frame to the carboxy-terminus of ubiquitin, proteins accumulate significantly in the plant cytoplasm. In addition artificial ubiquitin protein fusions resemble natural ubiquitin extension proteins, e.g. UBQ1 of *Arabidopsis thaliana* (Callis et al., 1990, J. Biol. Chem., 265, 12486-12493), in that they are cleaved precisely at the C-terminus of ubiquitin (after Gly 76 by specific endogenous proteases. This process releases the " attached" protein or peptide moiety from

the fusion protein and thus permits polypeptide A and B to assemble into a functional enzyme or protein. Also, for the purpose of protecting small proteins from cytoplasmic proteolysis, translational products may be enlarged by fusing them to protein targeting signals, e.g. the C-terminus (Whelan and Glaser, 1997, Plant Mol. Biol. 33, 771-781) and be directed to specific locations in the cell such as to mitochondria. A suitable signal, for example, is the one found in the AOX3 protein of soybean (Finnegan and Day, Plant Physiol., 1997, 114, pp 155) which would add 50 amino acids to polypeptide A and B, respectively. Import associated proteolytic processing will remove the targeting signal by cleavage after Met50 thereby releasing the free polypeptides A and B into the mitochondria where they combine to disrupt mitochondrial function and thus to compromise cell viability.

In some cases, when expressed in two or more portions, the polypeptides may not spontaneously associate, assemble, interact or come together *in vivo* to reform an active protein, or regulatory enzyme or protein which affects the structural integrity of a cell. In other cases the association of the polypeptides may be weak so that little active reconstituted protein is formed. To circumvent these problems, each protein portion may be linked to a protein dimerisation domain, thus enabling the portions to be brought together *in vivo*. Such protein dimerisation domains are found in many proteins that naturally form dimers or multimers and the linking technique is well known in the art.

For example, the human cysteine-rich protein LIM double zinc finger motif has been fused to the Ga14 and VP16

proteins. In contrast to the unmodified Ga14 and Vp16 proteins the LIM-Ga14 and LIM-VP16 associate *in vitro* and *in vivo* (in NIH 3T3 mammalian cells) forming an active transcription factor (Feuerstein *et al.*, 1994, Proc.Natl.Acad.Sci. U.S.A. 91, 10655-10659). The LIM motif is found in many organisms. For example, a sunflower pollen specific protein with a LIM domain has been identified (Batlz *et al.*, 1996, Plant Physiology (Supplement III, 59). Other protein dimerisation domains exist such as the leucine zipper (Turner, R. and Tijian R., 1989 Science, 243, 1689-1694), the helix-loop-helix (Murre *et al.*, 1989, Cell, 56, 777-783), the ankyrin Blank *et al.*, 1993, Trends in Biochemical Sciences, 17, 135-140) and the PAS (Huang *et al.*, 1993, Nature, 364, 259-262) domains.

One may also wish to ensure that the genes encoding polypeptides A or B are inserted in the genomes of parents a and b at an identical position (or at tightly linked positions) so that their chance of co-segregation in the transgenic hybrid is low. This can be advantageous, for example in the production of hybrid seed since the two genes that are used to create the male-sterile parent plant will subsequently segregate. Thus, F1 hybrid progeny are 100% male fertile since no hybrid plant can inherit both components of the male-sterility system.

The gene sequences carried by the parent plants a and b which encode part of the active enzyme, regulatory protein or protein which affects the structural integrity of a cell may be from a different organism. The gene sequences do not have to be plant derived and include genes from microbial or other sources. For

example, the gene sequences may be Arabidopsis endogenous sequences in maize or tomato parent plants. Also, the gene sequences may be those which, in combination with a tissue specific promoter, are expressed in a tissue in which the gene sequences are not normally expressed.

According to a second aspect of the invention there is provided a method for producing a plant having a desired phenotype of an active enzyme, a regulatory protein or a protein which affects the structural integrity of a cell, the method comprising crossing a first plant line with a second plant line wherein the first line contains one or more gene sequences encoding a polypeptide A which is part of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell but which line does not have the phenotype and wherein the second line contains one or more gene sequences encoding a polypeptide B which is complementary to the polypeptide or protein A but which line does not have the desired phenotype. Here, the term "complementary" means that when expressed in the same plant the polypeptides A and B associate, interact or come together to form the phenotype of an active enzyme, a regulatory protein or protein which affects the structural integrity of a cell.

Such a method may incorporate one or more of the features described above for the first aspect of the invention and the invention contemplates the application of these aspects according to the second aspect of the invention.

According to a third aspect of the invention there is

provided a seed or plant obtainable from a pair of plants according to the first aspect of the invention or by a method according to the second aspect of the invention.

According to a fourth aspect of the invention there is provided a seed or plant having a phenotype of an active enzyme, regulatory protein or protein which affects the structural integrity of the cell, which is caused by the combined action of two or more transgenes, the transgenes not being present on the same copy of a chromosome. The preferred embodiments of the first, second and third aspects of the invention also apply to the fourth aspect. This means that the two or more transgenes are either on different chromosomes, or on different copies of the same chromosome, ie. the plant is made by a cross.

The invention will now be described by the following non-limiting Examples:

EXAMPLE 1

Splitting the Barnase Gene into Two Components (Figure 1)

The results of Sancho and Ferscht, 1992, J.Mol.Biol., 224, 741-747 show that Barnase activity can be obtained by combining a peptide A containing amino acids 1 to 36 of the mature Barnase protein and peptide B containing amino acids 37 to 110 of the mature Barnase protein. The allele of Barnase which is described in Sancho and Ferscht is a mutant which has a methionine at position 36, allowing cyanogen bromide to cleave between 36 and

37 and produce the 2 peptides. The following genetic constructs, to express the peptides, were prepared:

Peptide A:

i. A Barnase gene with a methionine codon (amino acid position -1) added before codon 1 of the mature Barnase sequence so that translation can take place as described in Paul *et al*, 1992, Plant Mol.Biol., 19, 611-622.

ii. An ORF coding for a peptide called A*, containing a methionine followed by amino acids 1 to 35 of mature Barnase protein followed by an Ochre stop codon.

iii. A gene made of ORF A* under control of the A9 promoter by using IPCR on our plasmid p3079, which contains the AMS gene pA9-Barnase (as in i. above) - Barstar - CaMV 3' region. (See Figure 2).

Plasmid p3079 was constructed by cloning a fragment containing the ORFs for Barnase-Barstar, obtained by PCR from pWP127 (Paul *et al*, 1992, *supra*), in our plasmid p1415, which is a derivative of pWP91 (WO-A-9211379) where the EcoRV restriction site has been converted to HindIII. IPCR was then performed on p3079 using primers B3 and B4 (see Figures 1 and 2) designed so that the sequence between codon 36 of Barnase and stop codon of Barstar is not part of the amplified product. The IPCR amplified sequence was then circularised by ligation and the resulting plasmid was introduced into *E. coli* The plasmid was then prepared, cut with EcoRI and the fragment containing the ORF A* was cloned in the EcoRI sites of p1415, so that ORF A* would be under the

control of the A9 promoter from a sequence not treated by PCR. The resulting plasmid p2022 contains ORF A* in the A9 expression cassette.

iv. An ORF coding for a peptide called A**, comprised of a start methionine codon followed by amino acids 1 to 36 of the mutant Barnase (Sancho and Ferscht, 1992, supra) but lacking a stop codon.

This was obtained by PCR on template p2022 with primers B5 (retaining the XbaI site at the 5' end) and B6 generating a blunt 3' end.

v. A gene made of the translational fusion of ORF A** and the ORF of (Gly4 Ser)3/GUS under the control of the A9 promoter, the product of which shows peptide A fused in frame to the N-terminus of (Gly4 Ser)3/GUS (Figure 1D).

This was obtained by replacing the S-peptide ORF in plasmid p2028 (see example 3) with the ORF of plasmid A** (iv). For ORF replacement an IPCR was performed on plasmid p2028 using primers B7 (retaining the Xba site at the 5' end) and B8 (generating a blunt 3' end) to delete the region encoding the S-peptide from the S-peptide-GUS translational fusion. After digest with XbaI, the PCR fragment encoding peptide A** (iv) was inserted XbaI/blunt into the acceptor DNA generated by IPCR.

vi. An ORF coding for peptide A***, essentially identical to peptide A**(iv) but lacking a methionine start codon and containing an amber stop codon.

This was obtained by PCR on template p2022 using primers B9 (producing a blunt 5′ end) and B10 (introducing a BamHI site at the 3′ end). The 3′ end of the PCR product was digested with BamHI for construction of the ubiquitin-petide A*** translational fusion (below).

vii. A gene made of the translational fusion of genomic DNA encoding ubiquitin and the ORF A*** under the control of the A9 promoter, the product of which shows peptide A*** fused in frame to the C terminus of ubiquitin (Figure 1E).

The genomic DNA encoding ubiquitin was obtained by PCR from chromosomal DNA of *Arabidopsis thaliana*. The PCR primers Ubq16F and Ubq1R were designed to amplify the ubiquitin encoding sequence from the extension protein gene UBQ1, first described by Callis et al. (1990, supra). Restriction sites for XbaI (at 5′ end) and BamHI (at 3′ end), introduced during thermocycling, were used to clone the PCR fragment under the control of the A9 promoter of p1415 digested with XbaI and BamHI to yield plasmid p3245. IPCR was then performed on p3245 using primers UBQ1a and UBQ1b to generate a blunt acceptor end immediately after the ubiquitin codon Gly 76 and at the 3′ end to reconstitute the BamHI restriction site for cloning. After BamHI digest this construct served as acceptor for the PCR fragment encoding peptide A*** (vi).

Peptide B:

i. An ORF coding for a peptide called B* which starts with a methionine codon followed by codons 37 to 110 of the mature Barnase sequence. In effect this transfers the methionine 36 of the mutant Barnase gene (Sancho and

Ferscht, 1992; *supra*) from peptide A to peptide B, yielding peptides A* and B*.

ii. Gene for ORF B* containing the ATG (amino acid position -1) of Barnase (in p3079) fused to codon 37 of Barnase, under control of the A9 promoter, by deleting (by IPCR with suitable primers) (see below)) codons 1 to 36 of the mature Barnase sequence.

This was done by performing on p3079 an IPCR reaction using primers B1 and B2, (Figures 1 and 2) designed so that the sequence between codon 2 and codon 36 of Barnase is not part of the amplified product (see Figure 2). The IPCR product is treated as described above for ORF A*, and cloned under control of the A9 promoter in p1415. The resulting plasmid p2023 contains ORF B* - Barstar in the A9 expression cassette.

iii. An ORF encoding peptide B*** which differs from B* (i) in that it lacks the start methionine.

iv. A gene made of the translational fusion of genomic DNA encoding ubiquitin and the ORF B*** under the control of the A9 promoter, the product of which shows peptide B*** fused in frame to the C-terminus of ubiquitin (Figure 1F).

IPCR as performed on plasmid p2023 (above) with primers B11 and B12, retaining the XbaI site at the 5' end of B* but removing the ATG start and leaving a blunt 3' end. After digest with XbaI, the IPCR product served as an acceptor for the ubiquitin encoding DNA. The latter sequence was obtained by PCR from plasmid p3245 (above) with primers Ubq16F and Ubq1b retaining an Xbal site at

the 5' end while leaving the 3' end blunt. After digest with XbaI, the IPCR and the PCR product were ligated to yield the translational fusion shown.

In Figure 1G: The nucleotide sequences of primers are listed which were used for PCR and IPCR, respectively.

In Fig. 2: Circular plasmid p3079, containing the A9-driven barnase/barstar gene (Figure 1) in p1415, served as template for Inverse PCR. As the PCR primers (Figure 1) pointed into opposite directions, the IPCR yielded a linear double-stranded plasmid DNA from which the region in between the 5' ends of the annealed PCR primers was deleted (below). Intramolecular ligation would then yield circular deletion plasmids which were introduced into *E.coli* for further subcloning.

Also In Fig. 2-:
lane 1:
A schematic (not to scale) representation is shown of plasmid p3079. The different structural parts of the coding regions are highlighted. ATG and TAA represent the start and stop codon of barnase and barstar, respectively. The relative positions of codons 35, 36 and 37 of the mature Barnase protein are indicated.

lane 2:
IPCR with primers B1 and B2 deleted codons 1 to 36 of the mature Barnase protein. Intramolecular ligation of the linear deletion plasmid then fused the ATG start codon to codon 37 yielding the pepB*/barstar region.

lane 3:
IPCR with primers B3 and B4 deleted the sequence

downstream of the barnase codon 35 as indicated. Intramolecular ligation of the linear deletion plasmid then fused the barnase codon 35 to the barstar stop codon yielding the pepA* sequence.

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EXAMPLE 2

Plant Transformation with the Genetic Constructs in Example 1

Genes pA9-A* and pA9-B* expressing a polypeptide A and a polypeptide B from the A9 promoter (WO92/11379) were cloned into derivatives of the plant transformation vector pBin19 Beven *et al.*, 1984, Nucl. Ac. Res. 12, 8711 Frish *et al.*, 1995 Plant Mol. Biol., 27, 405-409 and Arabidopsis plants containing pA9-polypeptide A, or pA9-polypeptide B, or both genes, were obtained. Plants containing both genes were male sterile, whereas plants containing one gene were unaffected by the transgene. Plants with one gene were allowed to self, their progeny was harvested, and was analysed to identify homozygous and heterozygous T1 plants. T1 plants with pA9-polypeptide A were crossed with T1 plants with pA9-polypeptide B. The hybrid seeds obtained displayed the predicted phenotype: wild type if containing one gene only, and male sterile when containing the two genes.

Genes are introduced into maize and into tomato by biolistic or Agrobacterium-mediated transformation, and plants are regenerated and assessed for male fertility in a similar way.(Mornish *et al.*, 1990 Biol/Technology 8, 833-839 and Fillati *et al* ., 1987 Bio/Technology 5, 726-7390.

EXAMPLE 3

Splitting an RNAseA gene into two components (Figures 3 and 4)

From the work of Richards and Vithayathil (1959 *supra*), we know that the enzyme RNAseA can be cleaved (by the protease subtilisin) to generate two polypeptides: the S-peptide contains amino acids 1 to 20 of RNaseA, and the S-protein contains amino acids 21 to 124 of RNaseA. When combined, the S-peptide and the S-protein associate, and reconstitute an active enzyme. The last 5 amino acids of the S-peptide are not needed for reconstituting RNaseA: a smaller S-peptide made of amino acids 1 to 15 is sufficient. Genes which express the S-peptide and the S-protein under control of the A9 promoter were used to develop a system according to the invention.

The starting material was a synthetic gene coding for bovine pancreatic RNAseA (Vasantha and Filpula, 1989, Gene 76 53-60). A gene coding for the ORF of RNaseA was made using synthetic oligonucleotides (see Figures 3A and 3B). The nucleotide sequence of the gene was designed to be compatible with maize codon usage, according to Fennoy and Bailey-Serres, 1993 Nuc. Acids Res., 21, 5294-5300. PCR with suitable primers was used to amplify from the full length ORF. The following ORFs were built:

S-peptide:

i. An ORF for the S-peptide containing a methionine translation initiation codon followed by codons 1 to 15 of the mature RNaseA sequence (see Figures 4A and 4B) and terminated by an Ochre stop codon.

ii. An ORF made of a methionine translation initiation codon followed by codons 1 to 15 of the mature RNAseA sequence, followed by a linker sequence encoding (Gly4-

Ser)3 (see Figures 4A and 4B). This gene was designed so that it can be fused in frame to the ORF of the GUS protein by cloning in the BamHI site of plasmid p2027 which contains the GUS gene from pBI101.3 (Jefferson, 1987 Plant Mol.Biol.Reporter, 5 387-405).

iii. A translational fusion comprising the ORF of the mitochondrial protein targeting sequence of AOX3 protein from soybean (Finnegan and Day, 1997, Plant Physiol. 114, pp455) and the ORF of S-peptide as described in (i) but lacking the methionine translation initiation codon (Figure 4C). The gene product of said translational fusion shows the S-peptide fused to the C-terminal end of the targeting sequence.

iv. A translational fusion comprising the ORF of the mitochondrial protein targeting sequence of AOX3 protein (supra) and the ORF of the S-peptide-GUS fusion as described in (ii) but lacking the methionine translational initiation codon (Figure 4C). The gene product of said translational fusion shows that the S-peptide-GUS protein fused to the C-terminal end of the targeting sequence.

S-protein:

i. An ORF for the "S-protein +5", which contains a methionine translation initiation codon followed by codons 16 to 124 of mature RNAseA sequence and is terminated by an Ochre codon.

ii. An ORF for the S-protein which contains a methionine translation initiation codon followed by codons 21 to 124 of mature RNAseA sequence and is terminated by an Ochre codon.

iii. A translational fusion comprising the ORF of the mitochondrial protein targeting sequence of AOX3 protein (supra) and the ORF of the S-protein as described in (ii) but lacking the mathionine translational intitiation codon (Figure 4C). The gene product of said translational fusion shows the S-protein fused to the C-terminal end of ubiquitin.

iv. A translational fusion comprising genomic DNA encoding ubiquitin and the ORF of the S-protein as described in (ii) but lacking the methionine translational intitiation codon (Figure 4C). The gene product of said translational fusion shows the S-protein fused to the C-terminus of ubiquitin.

Genes under control of the A9 promoter were then built and introduced into plants as in Example 2.

In Fig. 3A: The sequences encoding the S-peptide, the S(+5)-protein and the S-protein were constructed by first aligning sense oligonucleotides RN-I to RN-VII lanes 2, 5, 7, 9, 11, 13, 16) along complementary guide oligonucleotides RN-1 to RN-6 (lanes 3, 6, 8, 10, 12, 14) and then selectively ligating the correctly aligned sense oligonucleotides using Taq-DNA-Ligase.

The ligation resulted in a continuous single DNA strand sense) which was subsequently amplified by Vent DNA polymerase (25 PCR cycles) using one of two primer pairs as follows: (i) Primers RN-a (lane 1) and RN-b (lane 15) amplified the full ligation product. The PCR product was gel purified and cleaved with restriction enzymes BamHI (underlined, lanes 1 and 15) and BgIII underlined,

lanes 2 and 4) to yield two DNA fragments encoding the S-peptide and the S(+5) protein. The two fragments were cloned separately into the BamHI site downstream of the pA9 promoter in plasmid p1415 to yield plasmids p4837 (S-peptide) and p4838 (S+5 protein). (ii) Primers RN-d (lane 4) and RN-b (lane 15) amplified the coding sequence of the S-protein. The PCR product was cloned as described in (i) to yield plasmid p4839 (S-protein).

lane 1: PCR primer (sense) RN-a
lane 2: Oligonucleotide RN-I and alignment to oligonucleotide RN-II
lane 3: Guide oligonucleotide RN-1 (antisense)
lane 4: PCR primer (sense) RN-d
lane 5: Oligonucleotide RN-II (continued from lane 2) and alignment to oligonucleotide RN-IIIN
lane 6: Guide oligonucleotide RN-2N (antisense)
lane 7: oligonucleotide RN-IIIN (continued from lane 5) and alignment to oligonucleotide RN-IV
lane 8: Guide oligonucleotide RN-3 (antisense)
lane 9: oligonucleotide RN-IV (continued from lane 7) and alignment to oligonucleotide RN-V
lane 10: Guide oligonucleotide Rn-4 (antisense)
lane 11: oligonucleotide RN-V (continued from lane 9) and alignment to oligonucleotide RN-VI
lane 12: Guide oligonucleotide RN-5 (antisense)
lane 13: oligonucleotide RN-VI (continued from lane 11 and alignment to oligonucleotide RN-VII
lane 14: Guide oligonucleotide Rn-6 (antisense)
lane 15: PCR primer (antisense) RN-b
lane 16: oligonucleotide RN-VII (continued from lane 13)

Symbols:
(5'): non-phosphorylated 5' end
(5P): phosphorylated 5' end
(30H): conventional 3' end
(small letters): bases added for the convenience of cloning.

In Fig. 3B: The sequences encoding the S-peptide with the $(Gly_4Ser)_3$-linker peptide were constructed by first aligning sense oligonucleotides RN-I and RN-VIII (lanes 2 and 4) along the complementory guide oligonucleotide RN-7, and then selectively ligating the correctly aligned oligonucleotides using Taq-DNA-Ligase.

The ligation resulted in a continuous single DNA strand which was subsequently amplified by Vent DNA polymerase (25 PCR cycles) using the primer pair RN-a (lane 1) and RN-c (lane 5). This PCR reaction yielded the full length, double stranded ligation product. The PCR product was gel purified, then cleaved with restriction enzymes BamHI (underlined, lane 1) and BglII (underlined, lane 5) and cloned into the BamHI site of p2027 to generate an $NH_2$-terminal protein fusion to GUS under the control of the pA9 promoter (p2027 was constructed by cloning the GUS coding sequence of pBI101.3 as a BamHI/SmaI fragment into the BamHI site of p1415). This yielded plasmid p2028.

lane 1: PCR primer (sense) RN-a
lane 2: Oligonucleotide RN-I encoding the S-peptide as in Figure 3a and the alignment to oligonucleotide RN-VIII encoding the (Gly4-Ser)3 linker peptide
lane 3: Guide oligonucleotide (antisense) RN-7

lane 4: Oligonucleotide RN-VIII (continued from lane 2)

lane 5: PCR primer (antisense) RN-c

Symbols:

(5'): non-phosphorylated 5' end

(5P): phosphorylated 5' end

(30H): conventional 3' end

(small letters): bases added for the convenience of cloning

In Figure 4A: The protein and DNA sequence is shown for S-peptide and the S-peptide with (Gly4 Ser)3 linker. The S-peptide linker sequence was fused in frame to GUS to yield plasmid p2028 as described for Figure 3B.

In Figure 4B: The ORF for (S+5)-protein and S-protein is shown as contained in plasmids p4838 and p4839, respectively. These plasmids were described above for Figue 3A.

In Figure 4C:

(i) The mitochondrial protein targeting sequence (short of the last four amino acids: Leu-Arg-Arg-Met) was obtained by PCR with primers AOX3MI1 and AOX3MI2 from a plasmid which contained the cDNA of Alternative Oxidase (AOX3) of soybean as published by Finnegan and Day, 1997 (Plant Physiol. 114, pp455). Restriction sites (XbaI and BglII at the 5'end and AflII and BamHI at the 3' end) were introduced during the thermocycling to yield the PCR product which was cloned XbaI/BamHI downstream of the A9 promoter in p1415. This plasmid was called p0200.

(ii) Primers SPEPMI1 and SPEPMI2 were then used to produce from plasmid p4837 a PCR fragment encoding within and downnstream of an AflII restriction site the missing four amino acids (Leu-Arg-Arg-Met) of the mitochondrial targeting signal followed by the ORF of S-peptide. A PCR generated BamHI site at the 3' end allowed cloning of the PCR fragment as an AflII/BamHI fragment into p0200. This cloning yielded plasmid p0203, containing the complete ORF of the translational fusion as shown.

(iii) The translational fusion of mitochondial targeting sequence and ORF of S-peptide-GUS was generated in a similar fashion as described in (ii) except that PCR primers SPEPMI1 and SPEPMI2 were used on template p2028 to generate an AflII/BamHI fragment that was cloned into p0200 to yield p0204.

(iv) The translational fusion of mitochondrial targeting sequence and ORF of S-protein was generated in a similar fashion as desccribed in (ii and iii), except that PCR primers SPROTMI1 and SPROTMI2 were used ontemplate p4838 to generate an AflII/BamHI fragment that was cloned into p0200 to yield p0202.

(v) A PCR fragment was generated from template p4839 with primers SPROTF and SPROTR containing the ORF of S-protein in between BamHI restriction sites at either end. After digestion with BamHI this PCR fragment was cloned into the BamHI site of p3245 which yielded the translational fusion in p3249 of genomic ubiquitin DNA and S-protein as shown.

EXAMPLE 4

Use of the Dimer Protein Apetala3-Pistillata

Apetala3 (Ape3) and Pistillata (Pi) are two proteins of *Arabidopsis thaliana* which are involved in the regulation of floral differentiation. The genes are known while the endogenous pattern of expression in the tapetum is not known. Expression of the Arabidopsis genes in the maize tapetum leads to disruption of the normal anther development by activating normally silent genes. These genes can also be used to activate, in the maize tapetum, an Arabidopsis promoter responsive to the Ap3-Pi dimer such as the Ap3 promoter (pAp3) itself.

We have built the following genes:

pA9-Apetala3

The cDNA for Ap3 (Jack *et al*, 1992, Cell 68, 683-697 GenBank Accession No. M86357) was cloned in the A9 expression cassette of pWP91 (WO-A-9211379) giving plasmid p4796. This plasmid contains the Ap3 cDNA with approximately 15 bases of 5' untranslated sequence followed by the whole ORF (698 bases from ATG to TAA) followed by approximately 120 bases of 3' untranslated sequence, cloned in the BamHI site of pWP91.

pA9-Pistillata

The cDNA for Pi (Goto and Meyerowitz, 1994, Genes Dev. 8, 1548-1560 GenBank Accession No. D30807) was cloned in the A9 expression cassette of pWP91 (WO-A-9211379) giving plasmid p0180. This plasmid contains the Pi cDNA with approximately 24 bases of 5' untranslated sequence followed by the whole ORF (626 bases from ATG to TGA) followed by approximately 250 bases of 3'untranslated

sequence, cloned in the XbaI-BamHI sites of pWP91.

pApetala3-PRGlucanase

The A9 promoter sequence in plasmid A9PR (described in Worrall *et al*, 1992, The Plant Cell, 4, 759-771) was replaced by a 1250 bp (approx) sequence containing the Ap3 promoter region, obtained by PCR amplification of *Arabidopsis thaliana* genomic DNA, according to the published sequence (Jack *et al*, 1994 Cell, 76, 703-716), giving plasmid p4817.

The genes were introduced in maize in various combinations, by biolistic transformation techniques known in the art. Plants were regenerated and assessed for male fertility.

-p4796 (pA9-Ap3)/p0180 (pA9-Pi) cause male sterility. Neither of them alone causes male sterility.

-p4796/p0180/p4817 (pAp3-PRGlucanase) cause sterility, when p4817 with only one of the two transcription factor genes does not.

CLAIMS

1. A pair of parent plants for producing seeds comprising:

(i) a first parent plant containing one or more gene sequences encoding a polypeptide or protein A: and

(ii) a second parent plant containing one or more gene sequences encoding a polypeptide or protein B;

wherein the polypeptides A, B, when expressed in separate plants, do not form an active enzyme, a regulatory protein or protein which affects the functionality and/or viability and/or the structural integrity of a cell, but when expressed in the same plant do form an active enzyme, regulatory protein, or protein which affects the structural integrity of a cell.

2. A pair of plants as claimed in claim 1 wherein the one or more gene sequences from at least one of the parents is transgenic.

3. A pair of plants as claimed in claim 1 or claim 2 wherein the polypeptides or proteins A, B, when expressed in the same plant, cause cell ablation, especially male-sterility or embryoless seeds.

4. A pair of plants as claimed in any one of claims 1 to 3 wherein one of the parent plants is male-sterile.

5. A pair of plants as claimed in any one of claims 2

to 4 wherein the one or more gene sequences encoding both or one of the polypeptides or proteins A, B, is operatively linked to a tissue specific promoter.

6. A pair of plants as claimed in any one of claims 1 to 5 wherein the polypeptides A, B are naturally occurring subunits of the protein complex of an active enzyme, regulatory protein, or protein which affects the structural integrity of a cell.

7. A pair of plants as claimed in claim 6 wherein the polypeptides A, B are two polypeptide subunits of an enzyme having RNase activity such as the enzyme Barnase or RNase A or the monomers of the protein complex of the Apelata3-pistillata.

8. A pair of plants as claimed in any one of claims 1 to 5 wherein the polypeptides A, B are artificially split polypeptides of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell.

9. A pair of plants as claimed in any one of the preceding claims wherein each parent plant is homozygous with respect to the one or more gene sequences encoding polypeptide A or B respectively.

10. A pair of plants as claimed in any one of claims 3 to 9 wherein the cause of male-sterility is direct or indirect.

11. A pair of plants as claimed in any one of claims 5 to 10 wherein the tissue-specific promoter is a tapetum-specific promoter, an embryo-specific promoter or a seed

specific promoter.

12. A pair of plants as claimed in any one of claims 1 to 11 wherein one or both of the polypeptides or proteins is fused to a carrier protein and/or a protein targeting signal.

13. A pair of plants as claimed in any one of claims 1 to 12 wherein each polypeptide or protein A, B is linked to a protein dimerisation domain of a dimeric or multimeric protein sequence that promotes association of between subunits A and B.

14. A pair of plants as claimed in any one of the preceding claims wherein the one or more gene sequences from at least one of the parent plants is a heterologous gene sequence.

15. A method for producing a plant having a desired phenotype by virtue of an active enzyme, a regulatory protein or a protein which affects the structural integrity of a cell, the method comprising crossing a first line with a second line wherein the first line contains one or more gene sequences encoding a polypeptide or protein but which line does not have the desired phenotype and wherein the second line contains one or more gene sequences encoding a polypeptide or protein B which is complementary to the polypeptide or protein A but which line does not have the desired phenotype.

16. A method as claimed in claim 15 wherein the one or more gene sequences from at least one of the lines is transgenic.

17. A method as claimed in claim 15 or claim 16 wherein desired phenotype is cell ablation especially male-sterility or embryoless seeds.

18. A method as claimed in any one of claims 15 to 17 wherein one of the lines is male-sterile.

19. A method as claimed in any one of claims 15 to 18 wherein the one or more gene sequences encoding polypeptides or protein A and/or B is operatively linked to a tissue-specific promoter.

20. A method as claimed in any one of claims 15 to 19 wherein the polypeptides or proteins A, B are naturally occurring subunits of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell.

21. A method as claimed in claim 20 wherein the polypeptides or proteins A, B are two polypeptide subunits of an enzyme having RNase activity such as the enzyme Barnase, RNase A or the subunits of the protein Apelata3-pistillata.

22. A method as claimed in any one of claims 15 to 19 wherein the polypeptides or proteins A, B are artificially split polypeptides of an active enzyme, regulatory protein or protein which affects the structural integrity of a cell.

23. A method as claimed in any one of claims 15 to 22 wherein each line is homozygous with respect to the gene sequence encoding polypeptide or protein A, B,

respectively.

24. A method as claimed in any one of claims 15 to 23 wherein the desired phenotypic trait is direct or indirect male-sterility.

25. A method as claimed in any one of the claims 15 to 24 wherein the tissue-specific promoter is a tapetum-specific promoter, an embryo-specific promoter or a seed specific promoter.

26. A method as claimed in any one of claims 15 to 25 wherein one or both of the polypeptides or proteins A, B is fused to a carrier protein and/or a protein targeting signal.

27. A method as claimed in any one of claims 15 to 26 wherein each polypeptide or protein A, B is linked to a different protein dimerisation domain of a dimeric or multimeric protein.

28. A method as claimed in any one of claims 15 to 27 wherein at least one of the lines contains, as the one or more gene sequences, heterologous gene sequences.

29. A seed or plant obtainable from a pair of plants as claimed in any one of claims 1 to 14 or by a method as claimed in any one of claims 15 to 28.

30. A seed or plant, having a phenotype of an active enzyme, regulatory protein or protein which affects the integrity of a cell, which is caused by the combined action of two or more transgenes, not present on the same copy of a chromosome.

# FIG. 1A

**Barnase coding sequence**

```
1                    met ala gln val ile asn thr phe asp gly val ala asp tyr leu gln thr tyr
2            TCTAGACC ATG GCA CAG GTT ATC AAC ACG TTT GAC GGG GTT GCG GAT TAT CTT CAG ACA TAT
3     3'gttcatgagatctgg tac 5' (B1 primer)

1                    his lys leu pro asp asn tyr ile thr lys ser glu ala gln ala leu gly trp
2                    CAT AAG CTA CCT GAT AAT TAC ATT ACA AAA TCA GAA GCA CAA GCC CTC GGC TGG
3                                                        (B4 primer)  3'  t gtt cgg gag ccg acc5'

1                    val ala ser lys gly asn leu ala asp val ala pro gly lys ser ile gly gly
2                    GTG GCA TCA AAA GGG AAC CTT GCA GAC GTC GCT CCG GGG AAA AGC ATC GGC GGA
3                      5'gca tca aaa ggg aac c 3' (B2 primer)

1                    asp ile phe ser asn arg glu gly lys leu pro gly lys ser gly arg thr trp
2                    GAC ATC TTC TCA AAC AGG GAA GGC AAA CTC CCG GGC AAA AGC GGA CGA ACA TGG
3

1                    arg glu ala asp ile asn tyr thr ser gly phe arg asn ser asp arg ile leu
2                    CGT GAA GCG GAT ATT AAC TAT ACA TCA GGC TTC AGA AAT TCA GAC CGG ATT CTT
3

1                    tyr ser ser asp trp leu ile tyr lys thr thr asp his tyr gln thr phe thr
2                    TAC TCA AGC GAC TGG CTG ATT TAC AAA ACA ACG GAC CAT TAT CAG ACC TTT ACA
3

1                    lys ile arg OCH
2                    AAA ATC AGA taa
3
```

# FIG. 1B

**Intergenic sequence**

```
CGAAAAAAACGGCTTCCTGCGGAGGCCGTTTTTTTCAGCTTTACATAAAGTGTGTAATAAATTTTTCTTCAAACTCTGATCGGTCAATTT
CACTTTCCGGATCCGGTCCAATCTGCAGCCGTCCGAGACAGGAGGACATCGTCCAGCTGAAACCGGGGCAGAATCCGGCCATTTCTGAAG
AGAAAAATGGTAAACTGATAGAATAAAATCATAAGAAAGGAGCCGCAC
```

# FIG. 1C

**Barstar coding sequence**

```
1                    Met lys lys ala val ile asn gly glu gln ile arg ser ile ser asp leu his
2                    ATG AAA AAA GCA GTC ATT AAC GGG GAA CAA ATC AGA AGT ATC AGC GAC CTC CAC
3

1                    gln thr leu lys lys glu leu ala leu pro glu tyr tyr gly glu asn leu asp
2                    CAG ACA TTG AAA AAG GAG CTT GCC CTT CCG GAA TAC TAC GGT GAA AAC CTG GAC
3

1                    ala leu trp asp cys leu thr gly trp val glu tyr pro leu val leu glu trp
2                    GCT TTA TGG GAT TGT CTG ACC GGA TGG GTG GAG TAC CCG CTC GTT TTG GAA_TGG
3

1                    arg gln phe glu gln ser lys gln leu thr glu asn gly ala glu ser val leu
2                    AGG CAG TTT GAA CAA AGC AAG CAG CTG ACT GAA AAT GGC GCC GAG AGT GTG CTT
3

1                    gln val phe arg glu ala lys ala glu gly cys asp ile thr ile ile leu ser
2                    CAG GTT TTC CGT GAA GCG AAA GCG GAA GGC TGC GAC ATC ACC ATC ATA CTT TCT
3

1                    OCH
2                    TAA TACGATCAATGGGAGATGAACAATATAGATCCCCCGGGCTGCAGGAATTC
3                  5'taa tacgatcaatgggagatg 3' (B3 primer)
```

1: Translation of DNA sequences encoding Barnase (A) and Barstar (C), respectively
2: DNA sequence encoding either Barnase (A), Barstar (C) or the synthetic intergenic region (B) according to Paul et al. (1992)
3: Sequence of DNA primers that were used for IPCR to construct pepA* (B3/B4) and pepB* (B1/B2).

# FIG. 1D

**Translational fusion of**
**ORF Peptide A**/ (Gly4 ser)3 Linker peptide / GUS**

```
        met ala gln val ile asn thr phe asp gly val ala asp tyr leu gln thr tyr his lys
tctagacc ATG GCA CAG GTT ATC AAC ACG TTT GAC GGG GTT GCG GAT TAT CTT CAG ACA TAT CAT AAG

        leu pro asp asn tyr ile thr lys ser glu ala gln ala leu gly trp met gly gly gly
        CTA CCT GAT AAT TAC ATT ACA AAA TCA GAA GCA CAA GCC CTC GGC TGG ATG GGC GGT GGC

        gly ser gly gly gly gly ser gly gly gly gly ser gly ile pro gly tyr gly gln ser
        GGT TCC GGT GGC GGT GGC AGC GGC GGC GGT GGT AGC GGg atc ccc ggg tac ggt cag tcc

        pro met
        ctt atg ... of GUS
```

Underlined: ORF of peptide A**

# FIG. 1E

**Nucleotide Sequence of Translational fusion of**
**Ubiquitin genomic sequence and ORF Peptide A*****

```
  tctagacc ATGCAGATCT TCGTGAAAAC CTTGACCGGC AAGACCATCA CTCTCGAGGT CGAGAGCAGC GACACCATCG
ACAATGTCAA GGCCAAGATC CAAGACAAAG AAGGTATCAT TCTTCCTCAC TCAATCTGGA TTCTTCTCTT TAGCTTTTTG
AAATTCAGAT CTCTTATCAT TTACTTGTTT CTCCTTTAAG GAATCCCTCC GGATCAGCAG AGATTGATCT TCGCCGGAAA
GCAGCTCGAA GATGGCCGTA CTTTGGCTGA CTACAACATC CAGAAAGGTA CGAAATCATC CGAATCCTTC TGTTGATCAT
TTCGATGATC TGATTGTATA AACTCTAATG GATTGTTATC ATTTGTAAAC AGAATCTACA CTTCATCTTG TGTTGAGGCT
TAGAGGTGGA GCACAGGTTA TCAACACGTT TGACGGGGTT GCGGATTATC TTCAGACATA TCATAAGCTA CCTGATAATT
ACATTACAAA ATCAGAAGCA CAAGCCCTCG GCTGGATGTA Gaggatcc
```

Underlined: Introns A and B within the ubiquitin sequence.
Bold: glycine codon 76 at the end of the ubiquitin ORF

# FIG. 1F

**Nucleotide Sequence of Translational fusion of Ubiquitin genomic sequence and ORF Peptide B*****

tctagacc ATGCAGATCT TCGTGAAAAC CTTGACCGGC AAGACCATCA CTCTCGAGGT CGAGAGCAGC GACACCATCG
ACAATGTCAA GGCCAAGATC CAAGACAAAG AAGGTATCAT TCTTCCTCAC TCAATCTGGA TTCTTCTCTT TAGCTTTTTG
AAATTCAGAT CTCTTATCAT TTACTTGTTT CTCCTTTAAG GAATCCCTCC GGATCAGCAG AGATTGATCT TCGCCGGAAA
GCAGCTCGAA GATGGCCGTA CTTTGGCTGA CTACAACATC CAGAAAGGTA CGAAATCATC CGAATCCTTC TGTTGATCAT
TTCGATGATC TGATTGTATA AACTCTAATG GATTGTTATC ATTTGTAAAC AGAATCTACA CTTCATCTTG TGTTGAGGCT
TAGAGGTGGA GCATCAAAAG GGAACCTTGC AGACGTCGCT CCGGGGAAAA GCATCGGCGG AGACATCTTC TCAAACAGGG
AAGGCAAACT CCCGGGCAAA AGCGGACGAA CATGGCGTGA AGCGGATATT AACTATACAT CAGGCTTCAG AAATTCAGAC
CGGATTCTTT ACTCAAGCGA CTGGCTGATT TACAAAACAA CGGACCATTA TCAGACCTTT ACAAAAATCA GATAA...

Underlined: Introns A and B within the ubiquitin sequence.
Bold: glycine codon 76 at the end of the ubiquitin ORF

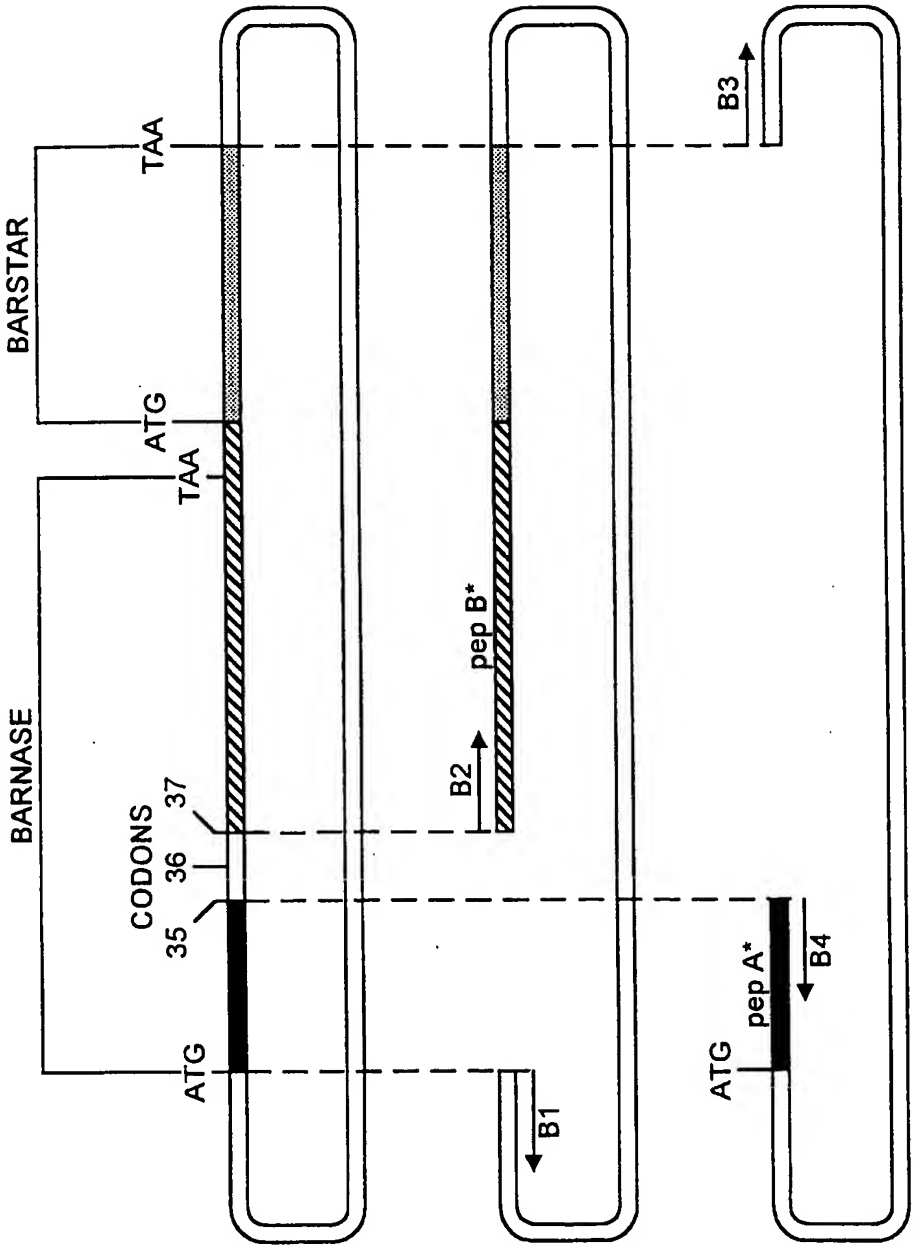
# FIG. 1G

**DNA sequence of T PCR primers (example 1)**

| | | |
|---|---|---|
| B5 | 5' CACAAGTACTCTAGACCATG 3' | (forward) |
| B6 | 5' CATCCAGCCGAGGGCTTGT 3' | (reverse) |
| B7 | 5' GGCGGTGGCGGTTCCG 3' | (forward) |
| B8 | 5' CCACTAGTTCTAGAGTACTTGTG 3' | (reverse) |
| B9 | 5' GCACAGGTTATCAACACG 3' | (forward) |
| B10 | 5' GCGGATCCTCTACATCCAGCCGAGGGCTTGT 3' | (reverse) |
| B11 | 5' GCATCAAAAGGGAACC 3' | (forward) |
| B12 | 5' GGTCTAGAGTACTTGTG 3' | (reverse) |
| Ubq16F | 5' GCTCTAGACCATGCAGATCTTCGTGAAAAC 3' | (forward) |
| Ubq1R | 5' CTGGATCCACCTCTAAGCCTCAACA 3' | (reverse) |
| Ubq1a | 5' TATGGATCCCCCGGGCTGCAGGAA 3' | (forward) |
| Ubq1b | 5' TCCACCTCTAAGCCTCAACAC 3' | (reverse) |

FIG. 2

SCHEMATIC ILLUSTRATION OF pepA* AND pepB* CONSTRUCTION BY INVERSE PCR (IPCR)



# FIG. 3A

## In Vitro Construction from Synthetic Oligonucleotides of S-peptide, S(+5)-protein and S-protein

```
1.   5'-gcggatccATGAAGGAGACCGCC-3OH
2.   5'-gcggatccATGAAGGAGACCGCCGCCGCCAAGTTCGAGCGCCAGCACATGGACAGC-3OH  5P-TAAAGATCTATG...
3.                                                                  3OH-GTACCTGTCG_____ATTTCTAGATAC-5'

4.   5'-ccagatctATG----AGCTCCTCCAACTACTG-3OH
5.   ...AGCACCTCCGCCGCCAGCTCCTCCAACTACTGCAACCAGATGATGAAGTCT-3OH  5P-AGGAACCTGA...
6.                                                          3OH-ACTACTTCAGA_____TCCTTGGACT-5'

7.   ...CCAAGGACAGGTGCAAGCCAGTCAACACCTTCGTCCACGAGAGCCTGGC-3OH  5P-CGATGTCCAG...
8.                                                          3OH-CTCGGACCG_____GCTACAGGTC-5'

9.   ...GCCGTCTGCAGCCAGAAGAACGTGGCCTGCAAGAACGG-3OH  5P-TCAGACCAACT...
10.                                    3OH-CGTTCTTGCC_____AGTCTGGTTGA-5'

11.  ...GCTACCAGTCCTACAGCACCATGTCCATCACCGACTGCCGCGAGACCGG-3OH  5P-CTCCAGCAAG...
12.                                                         3OH-GCTCTGGCC_____GAGGTCGTTC-5'

13.  ...TACCCTAACTGCGCCTACAAGACCACCCAGGCCAACAAGCACATC-3OH  5P-ATTGTTGCCTG...
14.                                              3OH-GTTCGTGTAG_____TAACAACGGAC-5'

15.                                       3OH-CTGCGGAGGCAGATTtcctagggc-5'
16.  ...CGAGGGTAACCCTTACGTGCCTGTCCACTTCGACGCCTCCGTCTAAaggatcccg-3OH
```

# FIG. 3B

## In Vitro Construction from Synthetic Oligonucleotides of the Sequence encoding the S-peptide and the (Gly4-Ser)3 Linker

```
1.  5'-gcggatccATGAAGGAGACCGCC-3OH
2.  5'-gcggatccATGAAGGAGACCGCCGCCGCCAAGTTCGAGCGCCAGCACATGGACAGC-3OH  5P-GGCGGTGG...;
3.                                                                 3OH-GTACCTGTCG_____CCGCCACC-5'

4.  ...CGGTTCCGGTGGCGGTGGCAGCGGCGGCGGTGGTAGCaagatcttcggg-3OH
5.                                 3OH-CCATCGttctagaagccc-5'
```

# FIG. 4A

**Protein and DNA Sequences of S-peptide and S-peptide with (Gly4 Ser)3 linker**

```
1     ------ --- AAA GAG ACA GCA GCC GCA AAG TTT GAG CGT CAG CAT ATG GAT AGT --- --- --- --- --- ---
2     ------ MET lys glu thr ala ala ala lys phe glu arg gln his met asp ser OCH gly gly gly gly ser
3     ggatcc atg aag GAG acc gcc GCC gcc AAG ttc GAG cgc CAG cac ATG gac agc taa --- --- --- --- ---
4     ggatcc atg aag GAG acc gcc GCC gcc AAG ttc GAG cgc CAG cac ATG gac agc --- ggc ggt ggc ggt tcc

1           --- --- --- --- --- --- --- --- --- --- ------
2           gly gly gly gly ser gly gly gly gly ser ------
3           --- --- --- --- --- --- --- --- --- --- -agatct
4           ggt ggc ggt ggc agc ggc ggc ggt ggt agc aagatct
```

Legend to Figure 4 A:

1: DNA sequence of the synthetic Bovine Rnase A gene (codon 1 to 15) according to N. Vasantha and David Filpula (1989)
2: Translation of synthetic DNA sequences encoding Bovine RNase A
3: DNA sequence of the S-peptide coding sequence referred to in this invention
4: DNA sequence encoding the S-peptide with (gly4 ser)3 linker peptide referred to in this invention

# FIG. 4B

**Protein and DNA Sequences of S(+5)-protein and S-protein**

```
1           --- AGC ACC AGT GCT GCC AGT TCT TCC AAC TAC TGT AAC CAG ATG ATG AAG TCT AGA AAC TTG ACC AAG
2           met ser thr ser ala ala ser ser ser asn tyr cys asn gln met met lys ser arg asn leu thr lys
3    agatct atg AGC ACC tcc gcc GCC agc tcc TCC AAC TAC tgc AAC CAG ATG ATG AAG TCT agg AAC ctg ACC AAG
4    agatct atg --- --- --- --- --- agc tcc TCC AAC TAC tgc AAC CAG ATG ATG AAG TCT agg AAC ctg ACC AAG

1              GAC AGA TGT AAG CCA GTT AAC ACA TTT GTC CAC GAG AGT TTG GCT GAT GTC CAA GCC GTC TGC AGT
2              asp arg cys lys pro val asn thr phe val his glu ser leu ala asp val gln ala val cys ser
3              GAC agg tgc AAG CCA gtc AAC acc ttc GTC CAC GAG agc ctg gcc GAT GTC cag GCC GTC TGC agc
4              GAC agg tgc AAG CCA gtc AAC acc tcc GTC CAC GAG agc ctg gcc GAT GTC cag GCC GTC TGC agc

1              CAG AAA AAC GTT GCA TGC AAG AAC GGT CAA ACG AAC TGT TAC CAG AGT TAC AGC ACC ATG TCC ATC
2              gln lys asn val ala cys lys asn gly gln thr asn cys tyr gln ser tyr ser thr met ser ile
3              CAG aag AAC gtg gcc TGC AAG AAC GGT cag acc AAC tgc TAC CAG tcc TAC agc ACC ATG TCC ATC
4              CAG aag AAC gtg gcc TGC AAG AAC GGT cag acc AAC tgc TAC CAG tcc TAC agc ACC ATG TCC ATC

1              ACT GAC TGT CGT GAG ACA GGC TCG AGC AAG TAT CCT AAT TGT GCT TAC AAG ACC ACA CAG GCG AAC
2              thr asp cys arg glu thr gly ser ser lys tyr pro asn cys ala tyr lys thr thr gln ala asn
3              acc GAC tgc cgc GAG acc GGC tcc AGC AAG tac CCT aac tgc gcc TAC AAG ACC acc CAG gcc AAC
4              acc GAC tgc cgc GAG acc GGC tcc AGC AAG tac CCT aac tgc gcc TAC AAG ACC ACA CAG gcc AAC

1              AAA CAC ATC ATT GTT GCT TGT GAA GGT AAC CCT TAC GTT CCT GTC CAC TTT GAC GCC AGT GTT TAA
2              lys his ile ile val ala cys glu gly asn pro tyr val pro val his phe asp ala ser val OCH
3              aag CAC ATC ATT GTT gcc tgc gag GGT AAC CCT TAC gtg CCT GTC CAC ttc GAC GCC tcc gtc TAA
4              aag CAC ATC ATT GTT gcc tgc gag GGT AAC CCT TAC gtg CCT GTC CAC ttc GAC GCC tcc gtc TAA

1              -------
2              -------
3              aggatcc
4              aggatcc
```

Legend to Figure 4 B:

1: DNA sequence of the synthetic Rnase A gene (codons 16 to 124) according to Vasantha and Filpula (1989)
2: Translation of DNA sequences encoding the Bovine RNase A
3: DNA sequence of the synthetic S(+5)-protein coding sequence (aa16 to aa124)
4: DNA sequence of the synthetic S-protein coding sequence (aa21 to aa124)

FIG. 4C



**i. PCR amplification product encoding impartial AOX3 targeting signal**

XbaI / BglII
tctagatcttaac ATGAAGAATG TTTTAGTAAG GTCAGCTGCG CGAGCTCTGC TTGGCGGCGG
TGGGCGGAGC TACTACCGCC AGCTCTCAAC GGCGGCGATC GTGGAACAGA
GACACCAGCA CGGTGGCGGC GCGTTTGGAA GCTTCCA cttaagcggatcc
AflII / BamHI

**ii. ORF encoding AOX3 targeting sequence (underlined) and S-peptide**

ATGAAGAATG TTTTAGTAAG GTCAGCTGCG CGAGCTCTGC TTGGCGGCGG TGGGCGGAGC
TACTACCGCC AGCTCTCAAC GGCGGCGATC GTGGAACAGA GACACCAGCA CGGTGGCGGC
GCGTTTGGAA GCTTCCACTT AAGAAGGATG AAGGAGACCG CCGCCGCCAA GTTCGAGCGC
CAGCACATGG ACAGCTAA

**iii. ORF encoding AOX3 targeting sequence (underlined) and S-peptide-(Gly4 Ser)3-GUS**

ATGAAGAATG TTTTAGTAAG GTCAGCTGCG CGAGCTCTGC TTGGCGGCGG TGGGCGGAGC
TACTACCGCC AGCTCTCAAC GGCGGCGATC GTGGAACAGA GACACCAGCA CGGTGGCGGC
GCGTTTGGAA GCTTCCACTT AAGAAGGATG AAGGAGACCG CCGCCGCCAA GTTCGAGCGC
CAGCACATGG ACAGCGGCGG TGGCGGTTCC GGTGGCGGTG GCAGCGGCGG CGGTGGTAGC
GGGATCCCCG GGTACGGTCA GTCCCTTATG --> GUS

**iv. ORF encoding AOX3 targeting sequence (underlined) and S-protein**

ATGAAGAATG TTTTAGTAAG GTCAGCTGCG CGAGCTCTGC TTGGCGGCGG TGGGCGGAGC
TACTACCGCC AGCTCTCAAC GGCGGCGATC GTGGAACAGA GACACCAGCA CGGTGGCGGC
GCGTTTGGAA GCTTCCACTT AAGAAGGATG AGCTCCTCCA ACTACTGCAA CCAGATGATG
AAGTCTAGGA ACCTGACCAA GGACAGGTGC AAGCCAGTCA ACACCTCCGT CCACGAGAGC
CTGGCCGATG TCCAGGCCGT CTGCAGCCAG AAGAACGTGG CCTGCAAGAA CGGTCAGACC
AACTGCTACC AGTCCTACAG CACCATGTCC ATCACCGACT GCCGCGAGAC CGGCTCCAGC
AAGTACCCTA ACTGCGCCTA CAAGACCACA CAGGCCAACA AGCACATCAT TGTTGCCTGC
GAGGGTAACC CTTACGTGCC TGTCCACTTC GACGCCTCCG TCTAA

**v. Translational fusion of Ubiquitin genomic sequence and ORF of S-protein**

ATGCAGATCT TCGTGAAAAC CTTGACCGGC AAGACCATCA CTCTCGAGGT CGAGAGCAGC
GACACCATCG ACAATGTCAA GGCCAAGATC CAAGACAAAG AAGGTATCAT TCTTCCTCAC
TCAATCTGGA TTCTTCTCTT TAGCTTTTTG AAATTCAGAT CTCTTATCAT TTACTTGTTT
CTCCTTTAAG GAATCCCTCC GGATCAGCAG AGATTGATCT TCGCCGGAAA GCAGCTCGAA
GATGGCCGTA CTTTGGCTGA CTACAACATC CAGAAAGGTA CGAAATCATC CGAATCCTTC
TGTTGATCAT TTCGATGATC TGATTGTATA AACTCTAATG GATTGTTATC ATTTGTAAAC
AGAATCTACA CTTCATCTTG TGTTGAGGCT TAGAGGtGGa tcCagCTCCA ACTACTGCAA
CCAGATGATG AAGTCTAGGA ACCTGACCAA GGACAGGTGC AAGCCAGTCA ACACCTCCGT
CCACGAGAGC CTGGCCGATG TCCAGGCCGT CTGCAGCCAG AAGAACGTGG CCTGCAAGAA
CGGTCAGACC AACTGCTACC AGTCCTACAG CACCATGTCC ATCACCGACT GCCGCGAGAC
CGGCTCCAGC AAGTACCCTA ACTGCGCCTA CAAGACCACA CAGGCCAACA AGCACATCAT
TGTTGCCTGC GAGGGTAACC CTTACGTGCC TGTCCACTTC GACGCCTCCG TCTAA

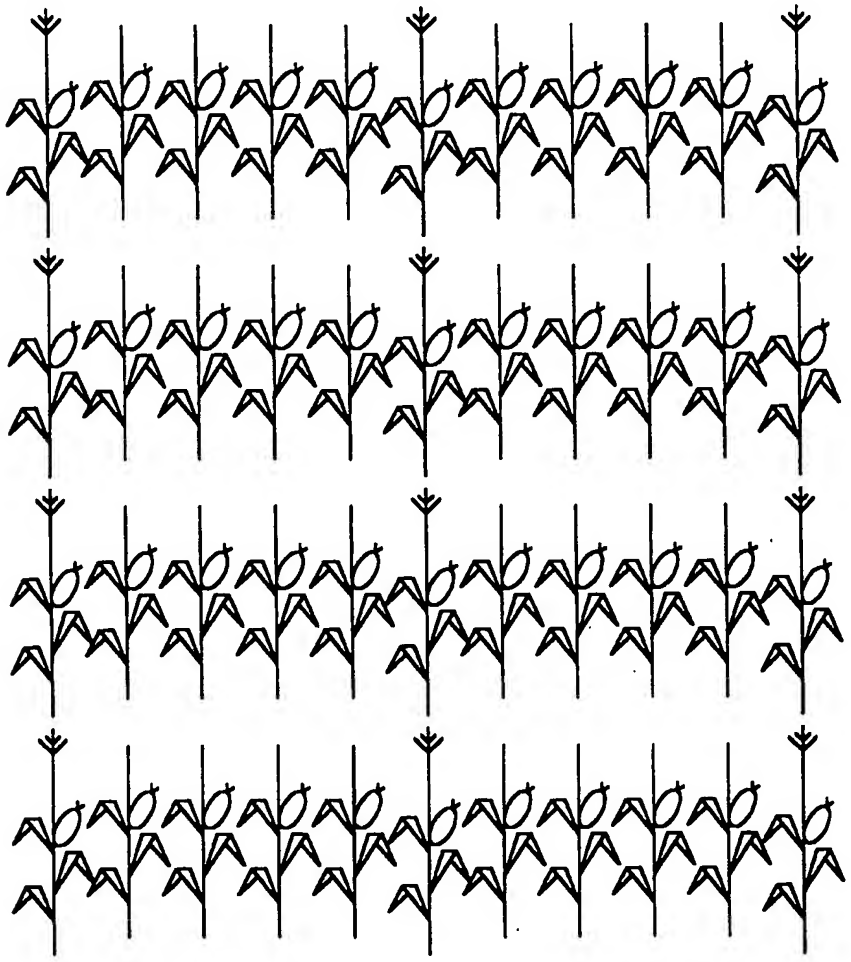
Underlined: introns A and B within the ubiquitin encoding sequence
Bold: codon for Glycine$^{76}$, marking the C-terminus of the ubiquitin.
Small letters: PCR introduced conservative codon changes to generate a BamHI site and to modify the codon usage

FIG. 4D

**Nucleotide sequence of T PCR primers (example 3)**

| | |
|---|---|
| Sprot F | 5' GGTGGATCCAGCTCCAACTACTGCAAC 3' |
| Sprot R | 5' CGGGATCCTTAGACGGAGGCGTCG 3' |
| SprotMI1 | 5' GTCCTTAAGAAGGATGAGCTCCTCCAACTAC 3' |
| SprotMI2 | 5' CGGGATCCTTAGACGGAGGCGTCG 3' |
| SpepMI1 | 5' GTCCTTAAGAAGGATGAAGGAGACCGCCG 3' |
| SpepMI2 | 5' TCGGGATCCTTAGCTGTCCATGTGCTG 3' |
| SpepGMI2 | 5' TCGGGATCCTCATTGTTTGCCTCCCTG 3' |
| AOX3MI1 | 5' TGCTCTAGATCTTAACATGAAGAATGTTTTAG 3' |
| AOX3MI2 | 5' TCGGATCCGCTTAAGTGGAAGCTTCCAAAC 3' |

FIGURE SHOWING A PRODUCTION SCHEME OF EMBRYO LESS MAIZE GRAINS: LINES A AND B ARE SOWN IN ALTERNATIVE ROWS (FOR EXAMPLE ONE MALE AND FOUR FEMALES)



LEGEND
(REFER TO DESCRIPTION FOR DETAILS)

MALE PARENT A

FEMALE PARENT B

FIG. 5

# INTERNATIONAL SEARCH REPORT

national Application No
PCT/GB 98/00542

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N9/22 C12Q1/68 A01H5/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 96 40950 A (PIONEER HI BRED INT) 19 December 1996<br><br>PAGES 7,17,18,41,42,46,52,66-68; CLAIMS<br>--- | 1-3,5, 9-11, 14-17, 19, 23-25, 28-30 |
| X | WO 96 04393 A (US GOVERNMENT ;DELTA & PINE LAND CO (US)) 15 February 1996<br><br>PAGES 3, LINE 3-7; 4, 6-7,8,14; EXAMPLES 1,3,7,9,10<br>---<br>-/-- | 1-5, 9-11, 14-19, 23-25, 28-30 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of theinternational search | Date of mailing of the international search report |
|---|---|
| 25 June 1998 | 09/07/1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Holtorf, S |

2

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# INTERNATIONAL SEARCH REPORT

national Application No
PCT/GB 98/00542

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 95 20668 A (GENE SHEARS PTY LTD ;BETZNER ANDREAS (AU); HUTTNER ERIC (AU); PERE) 3 August 1995 cited in the application<br><br>PAGES 1,2,15,27; EXAMPLES 5,9 | 1-3,5, 9-11, 14-19, 23-25, 28-30 |
| X | EP 0 589 841 A (CIBA GEIGY AG) 30 March 1994<br><br>ABSTRACT, PAGES 3, LINE 26-58; PAGE 5, PAGE 6,LINE 18-35; PAGE 7; EXAMPLES | 1-3,5, 9-11, 14-17, 19,23, 25,28-30 |
| A | SANCHO,J. AND FERSHT,A.R.: "DISSECTION OF AN ENZYME BY PROTEIN ENGINEERING" JOURNAL OF MOLECULAR BIOLOGY, vol. 224, 1992, pages 741-747, XP002068809 cited in the application see the whole document | 1-30 |
| P,X | WO 97 40179 A (PIONEER HI BRED INT) 30 October 1997<br><br>PAGE 4,9; EXAMPLE 4 | 1-5,11, 14-19, 23,25, 28-30 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

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Information on patent family members

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PCT/GB 98/00542

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| | | BR 9508471 A | 28-10-1997 |
| | | CA 2196410 A | 15-02-1996 |
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